PATENT #15
BD
2-13-03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Michael Spencer, Rita Mumm,
J. Jefferson Gwynn, David McElroy and
Michael A. Stephens

Serial No.: 09/698,789

Filed: October 27, 2000

For: METHOD FOR PLANT BREEDING (AS AMENDED)

Group Art Unit: 1638

Examiner: Kruse, David H.

Atty. Dkt. No.: DEKM:157USC1

CERTIFICATE OF MAILING
37 C.F.R. § 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on the date below:

01/31/03 Date

Robert E. Hanson

DECLARATION OF DR. PAUL FENG UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

I, PAUL C.C. FENG , HEREBY DECLARE AS FOLLOWS:

1. I am currently employed by Monsanto Company, the parent company of DeKalb Genetics Corporation, with the title of Research Scientist. I have a Ph.D. in Biochemistry from North Dakota State University. I have been conducting research in the area of agricultural biotechnology for over 21 years. I am the author of 46

manuscripts in peer-reviewed journals and a frequent speaker at national meeting in areas of weed science, plant physiology and agricultural biotechnology.

2. I am familiar with the subject matter disclosed and claimed in the above-referenced patent application.

3. I understand that the Patent and Trademark Office Examiner in charge of assessing the patentability of the referenced patent application has rejected the claims as not being supported by adequate information in the specification regarding the ability to produce transgenes that confer a glyphosate-inducible male sterile phenotype in maize.

4. Therefore, I am providing the present Declaration to submit further data that demonstrate the enablement of the invention claimed in the current patent application.

5. Studies carried out under my supervision have shown the broad applicability of the use of glyphosate-resistant EPSPS coding sequences to engineer plants exhibiting vegetative tolerance and male reproductive sensitivity to glyphosate (glyphosate-inducible male sterility). These studies were initiated with the goal of obtaining plants having sufficient expression of glyphosate-resistant EPSPS in vegetative tissues to provide vegetative tolerance to glyphosate, but having little or no expression in male reproductive parts, so that the plants exhibit a glyphosate-inducible male sterile phenotype. This strategy is set forth on pages 78 and 79 of the above-referenced patent application. There it is indicated, and our studies have confirmed, that the glyphosate-inducible phenotype is due to this expression profile.

6. Histological studies carried out on transgenic maize at Monsanto Company have shown that glyphosate arrests the maturation of microspore pollen cells, resulting in inviable pollen and male sterility. The studies indicate that the impact of glyphosate is focused at specific stages of pollen development; during the development of the microspore mother cell, tetrad, and microspores. Experiments indicate that, once microspores begin to mature (~V14 stage), glyphosate is no longer effective.

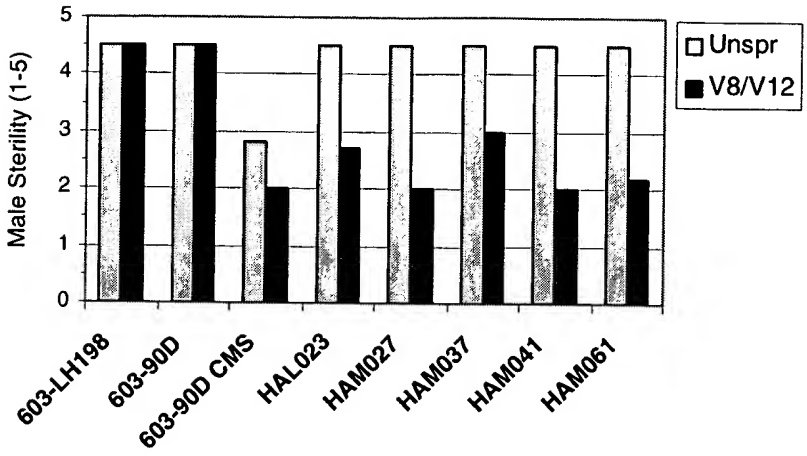
Studies carried out by myself and others at Monsanto Company have demonstrated that pollen with little or no expression of a glyphosate-resistant EPSPS transgene is susceptible to glyphosate, whereas pollen expressing high levels of resistant EPSPS is not. In particular, immunolocatization studies carried out at Monsanto Company demonstrated that male fertile glyphosate-resistant plants display high expression of glyphosate-resistant EPSPS expression in the tapetum, microspore mother cell, tetrad and microspores. In contrast, plants exhibiting vegetative glyphosate tolerance and male reproductive intolerance (glyphosate-inducible male sterility), display low to no expression in the same tissues. These results demonstrate that the inducible male-sterile phenotype is dependent on the level of expression of glyphosate-resistant EPSPS in these male tissues. This is further supported, for example, by the description of others of glyphosate-induced male sterility in cotton (U.S. Patent Application No. 20020111272) and the ability to render pollen inviable with the herbicide chlorsulfuron in *Brassica napus* plants (U.S. Patent No. 6,476,291) .

7. The *de novo* creation and analysis of transgenic plants having a glyphosate-inducible male sterile phenotype that was carried out under my supervisions can be summarized as follows:

Engineering of glyphosate-inducible male sterility:

Five homozygous transformation events (HALO23, HAM37, HAM027, HAM041, HAM061) were prepared using a transgene construct comprising an expression-optimized CaMV 35S (CaMVe35S) upstream of a non-translated leader sequence from *Petunia hybrida* (hsp70) linked to a glyphosate-tolerant EPSPS from *Agrobacterium tumefaciens* (CP4 EPSPS). Plants homozygous for each transformation event were examined for glyphosate-induced male sterility and yield in 6 locations. Controls contained the NK603 transformation event in corn line LH198 (603-LH198) and 90DJD28 (603-90D) backgrounds as well as a NK603-CMS (cytoplasmic male sterile) line in the 90DJD28 (603-90D CMS) background. The NK603 transformation event comprises a CP4 EPSPS coding sequence and confers vegetative and male reproductive tolerance to glyphosate. LH198 and 90DJD28 are proprietary inbred maize lines of Monsanto Company.

Five treatments were carried out on the plants: unsprayed, hand detasseled by removal of 3 top leaves, V8 spray at 0.75 lb/a glyphosate, V12 spray at 0.56 lb/a, and a double spray at V8fbV12. All plants were treated with a V4 spray of 0.48 lb/a to insure that they were resistant to glyphosate. A comparison of the male sterility rating of the unsprayed control and the double sprays (V8fbV12) is presented in the figure below. Full male sterility with no anther extrusion is achieved at a rating scale of 2, while full fertility is achieved at a rating of 5.



The results showed that both NK603 controls (603-LH198 and 603-90DJD28) were fully male fertile (4.5 to 5.0 score) with the double spray. The NK603-CMS line in 90DJD28 showed incomplete male sterility (2.8 score) as expected having the CMS trait, for which sterility was improved by the double glyphosate spray treatment. Three out of the 5 events prepared with the e35S/hsp70/CP4 transgene (HAM027, HAM041, HAM061) showed glyphosate-induced full male sterility (2.0 score), while the remaining two events showed incomplete male sterility. Single sprays (V8 or V12) showed pollen shed at 90% silking, suggesting that a higher rate may be needed to generate full male sterility from a single spray in late stage development. These results demonstrated that all of the CaMVe35S/hsp70/CP4 EPSPS events examined in this study exhibited a glyphosate effect on male sterility, three of five events exhibited complete sterility. The results confirm the ability to reproducibly generate glyphosate-inducible male sterile plants, as evidenced by the three events exhibiting full glyphosate-induced male sterility. These results are representative of a larger body of events that demonstrated glyphosate-induced male sterility.

Inbred genotype performance. The 5 events examined above were backcrossed (2x) into 4 different genotypes (87DIA4, LH59, LH195, and LH198). The ability to induce male sterility was evaluated by applications of a single spray from V6-V12 at 0.56-0.75 lb/a glyphosate. The corresponding NK603 inbred in the same genetic background was used as the control. Acceptable male sterility (2.0-2.5 score) was observed in the 87DIA4 background from application at V10/0.56 lb/a glyphosate. In LH59, 4/5 events showed acceptable male sterility from V10/0.56 lb/a. For LH195 and LH198, male sterility was observed at either V10/0.56 or V12/0.56 lb/a. The corresponding treatments for NK603 inbreds were all fertile. Greenhouse evaluations of plants at the F1 stage also showed good male sterility in other backgrounds, including FBLL, LH172, LH244, and LH295.

The results obtained demonstrate that glyphosate-inducible male sterility works in multiple genotypes. The results show that the observed inducible male sterile phenotype was not due to genetic background, based on the observation of glyphosate-induced male sterility in all 4 genotypes. The results further demonstrated the ability to introduce the glyphosate-inducible male sterile phenotype into multiple genetic backgrounds.

8. Based on the identified expression profile for creation of glyphosate-inducible male-sterile plants, an analysis was carried out to identify a sampling of transformation constructs for ready generation of the herbicide-inducible male sterile phenotype. Addiitonal constructs were prepared containing these promoters and the linked genetic elements that have demonstrated an expression profile that is high in vegetative tissue and low in male reproductive tissue and are described in Tables 1 below. Transgenic corn plants were produced that contained these constructs and were field tested for male sterility after glyphosate application. Events from each

of the constructs listed in Table 1 have demonstrated vegetative glyphosate tolerance and male sterility.

Table 1. DNA constructs that provide a male sterile phenotype in corn.

Constructs	Code	Genetic Elements
PMON58400	HAL	CaMVe35S/hsp70 intron/CP4 EPSPS-1 /nos 3'
PMON58401	HAM	CaMVe35S/hsp70 intron/CP4 EPSPS-2/nos 3'
PMON42471	HAD	CaMV35Schimeric1/hsp70 intron/CP4 EPSPS-1/nos 3'
PMON42475	HAH	CaMVe35S.ract1/ hsp70 intron/CP4 EPSPS-1/nos 3'
PMON42469	HAB	CaMVe35S/rss intron/CP4 EPSPS-1/nos 3'
PMON42476	HAI	CaMV35Schimeric2/hsp70 intron/CP4 EPSPS-1/nos 3'
PMON42474	HAG	CaMV35Schimeric3/hsp70 intron/CP4 EPSPS-1/nos 3'

9. It is my opinion, based on the studies above, the teachings in the current application, the working examples describing the creation of plants exhibiting a glyphosate-inducible male sterile phenotype, the description of the expression profile for creation of such plant, the knowledge of one of skill in the art at the time the application was filed, and my experience in agricultural biotechnology, that one of skill in the art in possession of the patent current application could readily prepare transgenic maize plants with a glyphosate-inducible male sterile phenotype using many different combinations of promoters and glyphosate resistant EPSPS transgenes without undue experimentation.

10. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Jan 15, 2003
Date

Paul C. C. Feng
Paul C.C. Feng

25243789_1111.DOC